Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Pseudorabies Virus Neutralizing Antibody
(Constant Virus - Varying Serum Method)

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Supplemental Assay Method for the Titration of
Pseudorabies Virus Neutralizing Antibody

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1. Introduction

This is an *in vitro* serum neutralization (SN) assay method which utilizes cytopathic effects (CPE) or fluorescent antibody technique (FAT) in a cell culture system to determine the SN antibody titer against pseudorabies virus (PRV). The SN assay uses a constant amount of virus to test varying dilutions of serum. The assay meets the requirements in the Code of Federal Regulations, Title 9 (9 CFR) to test serum samples collected from vaccinated and control swine for potency testing of inactivated PRV vaccines.

2. Materials

2.1 Equipment/Instrumentation

2.1.1 $36^0 \pm 2^0$C, 5 ± 1% CO_2, high humidity incubator[1] meeting the requirements in the current version of GDOCSOP0004

2.1.2 Water bath[2]

2.1.3 Inverted light microscope[3]

2.1.4 Fluorescent microscope[4]

2.1.5 96-well cell culture plates[5]

2.1.6 Vortex mixer[6]

2.1.7 Micropipetters: 200-μl and 1000-μl single channel;[7] 300-μl x 12-channel[8]

2.1.8 12x75-mm polystyrene tubes[9]

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[1] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent

[2] Cat. No. 15-461-10, Fisher Scientific, Inc., 319 West Ontario, Chicago, IL 60610 or equivalent

[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent

[4] Model BH2, Olympus America, Inc. or equivalent

[5] Costar 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent

[6] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent

[7] Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent

[8] Finnpipettes, Cat. No. NX204662D, A. Daigger Company, Inc., 199 Carpenter Ave., Wheeling, IL 60090 or equivalent

[9] Falcon 2058, Becton Dickinson Labware, Becton Dickinson & Co., 2 Bridgewater Ln., Lincoln Park, NJ 07035 or equivalent

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2.2 Reagents and Supplies

2.2.1 PRV reference virus, Shope strain[10]

2.2.2 Madin-Darby bovine kidney[11] (MDBK) cells free of extraneous agents as tested by 9 CFR

2.2.3 Minimum Essential Medium (MEM)

2.2.3.1 9.61 g MEM[12]

2.2.3.2 2.2 g sodium bicarbonate[13]

2.2.3.3 Q.S. to 1000 ml with deionized water, adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl)[14]

2.2.3.4 Sterilize through 0.22-μm filter.[15]

2.2.3.5 Aseptically add:

1. 10 ml L-glutamine[16]

2. 5 ml lactalbumin hydrolysate[17]

3. 100 units/ml penicillin[18]

4. 50 μg/ml gentamicin sulfate[19]

5. 100 μg/ml streptomycin[20]

2.2.3.6 Store at $4^0 \pm 2^0$C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

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[10] Reference virus, available on request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010

[11] Cat. No. CCL-22, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776 or equivalent

[12] MEM with Earle's salts without sodium bicarbonate, Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

[13] Cat. No. S 5761, Sigma Chemical, Inc., P.O. Box 14508, St. Louis, MO 63178 or equivalent

[14] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

[15] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

[16] L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies or equivalent

[17] Edamin S, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

[18] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent.

[19] Cat. No. 0061-0464-04, Schering Laboratories or equivalent

[20] Cat. No. S-9137, Sigma Chemical, Inc. or equivalent

2.2.4.2 Aseptically add 100 ml fetal bovine serum (FBS), heat inactivated at 56⁰ ± 2⁰C for 30 ± 5 minutes.

2.2.5 Maintenance Medium

2.2.4.1 98 ml of MEM

2.2.5.2 2 ml of FBS

2.2.6 Swine Anti-Pseudorabies Fluorescein Isothiocyanate Labeled Conjugate[21]

2.2.7 Dulbecco's phosphate buffered saline (D-PBS)

2.2.7.1 8.0 g sodium chloride (NaCl)[22]

2.2.7.2 0.2 g potassium chloride (KCl)[23]

2.2.7.3 0.2 g potassium phosphate, monobasic, anhydrous (KH_2PO_4)[24]

2.2.7.4 0.1 g magnesium chloride, hexahydrate ($MgCl_2 \bullet 6H_2O$)[25]

2.2.7.5 Dissolve with approximately 900 ml deionized water to obtain a primary solution.

2.2.7.6 Dissolve 1.03 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)[26] with 10 ml deionized water. Heat to 60⁰ ± 2⁰C, then add to primary solution.

2.2.7.7 Dissolve 0.1 g calcium chloride ($CaCl_2$)[27] with 10 ml deionized water. Add slowly to the primary solution to avoid precipitation.

2.2.7.8 Q.S to 1000 ml with deionized water, adjust pH to 7.0-7.3 with 2N HCl, and filter through a 0.22-μm filter.

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[21] PRV conjugate, available on request from the CVB-L or equivalent

[22] Cat. No. 3624-01, J.T. Baker or equivalent

[23] Cat. No. P217-500, Fisher Scientific, Inc. or equivalent

[24] Cat. No. 3246-01, J.T. Baker or equivalent

[25] Cat. No. M33-500, Fisher Scientific, Inc. or equivalent

[26] Cat. No. 3828-01, J.T. Baker or equivalent

[27] Cat. No. 4225-05, J.T. Baker or equivalent

2.2.8 80% Acetone

2.2.8.1 80 ml acetone[28]

2.2.8.2 20 ml distilled water

2.2.8.3 Store at room temperature (RT) (20^0 to 25^0C).

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in the immunologic basis of SN assays, cell culture techniques, and FAT and in the principles of aseptic technique.

3.2 Preparation of equipment/instrumentation

3.2.1 Set a water bath at $56^0 \pm 2^0$C.

3.2.2 Set a water bath at $36^0 \pm 2^0$C.

3.3 Preparation of reagents/controls

3.3.1 Two days prior to test performance

3.3.1.1 Seed 96-well cell culture with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 2 days of incubation at $36^0 \pm 2^0$C. This becomes the MDBK Plate. Growth Medium is changed if excess acidity of the medium is observed as indicated by a change from red to yellow of Growth Medium or cells are not confluent 2 days after incubation.

3.3.2 On day of test performance

3.3.2.1 Stock Virus Preparation. Rapidly thaw a vial of PRV Reference Virus in a $36^0 \pm 2^0$C water bath. Dilute the virus in MEM to contain 50-300 50% tissue culture infective dose ($TCID_{50}$)/100 μl.

[28] Cat. No. A 6015, Sigma Chemical, Inc. or equivalent

3.3.2.2 Virus Back Titration. Make 3 serial tenfold dilutions of Stock Virus.

1. Place 0.9 ml of MEM into 3, 12x75-mm polystyrene tubes labeled 10^{-1} to 10^{-3} respectively.

2. Transfer 0.1 ml of Stock Virus to the 10^{-1} tube; mix by vortexing. Discard pipet tip.

3. Transfer 0.1 ml from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing. Discard pipet tip.

4. Repeat **3.3.2.2.3** to the remaining tube, transferring from the 10^{-2} to the 10^{-3} tube.

3.3.2.3 On day of MDBK Plate examination

1. Dilute Swine Anti-pseudorabies Fluorescein Isothiocyanate Labeled Conjugate according to manufacturer's instructions.

3.4 Preparation of the sample (on day of test performance)

3.4.1 Heat inactivate all Test Serum Samples in a $56^0 \pm 2^0$C water bath for 30 ± 5 minutes.

3.4.2 Prepare serial twofold dilutions of Test Serum Samples in a 96-well cell culture plate, which becomes the Dilution Plate (**see Appendix I**). Twofold dilutions are made as follows:

1. Add 150 μl MEM to all wells in Rows B-H.

2. Add 150 μl Test Serum Samples to Rows A and B. Mix Row B with the multi-channel micropipetter (4-5 fills). The same tips may be used throughout.

3. Transfer 150 μl from Row B to Row C. Mix Row C with the multi-channel micropipetter (4-5 fills).

4. Continue as in **3.4.2.3** for the remaining rows. Discard 150 μl from all wells in Row H.

5. Add 150 μl of Stock Virus to all wells of the Dilution Plate. Tap plates gently to mix. Incubate for 60 ± 10 minutes at 36^0 ± 2^0C to allow for neutralization of virus. This is an additional twofold dilution of Test Serum Samples.

4. Performance of the test

4.1 Decant Growth Medium from a MDBK Plate.

4.2 Inoculate 50 μl/well of each Virus-Test Serum mixture into 5 wells/dilution of a MDBK Plate.

4.3 Inoculate 50 μl of each dilution of Virus Back Titration into 5 wells of a MDBK Plate.

4.4 Maintain 2 or more wells on a MDBK Plate as uninoculated cell controls.

4.5 Incubate MDBK Plates for 60 ± 10 minutes at 36^0 ± 2^0C.

4.6 Add 200 μl/well of Maintenance Medium to all wells (do not remove virus serum mix). Incubate MDBK Plate for 96 ± 12 hours postinoculation (HPI) at 36^0 ± 2^0C.

4.7 CPE counting is the primary method of determining the $TCID_{50}$.

4.7.1 96 HPI, examine the wells with an inverted microscope. The CPE of PRV is visible as grape-like clusters of rounded cells in the cell monolayer where the cells have been destroyed by the virus.

4.7.2 Record the number of wells/dilution showing any characteristic CPE of PRV for each Test Serum Sample and Virus Back Titration.

4.7.3 Calculate the $TCID_{50}$ of the Test Serum Samples and Virus Back Titration using the Spearman-Kärber method as modified by Finney.

4.7.4 The titer of the Test Serum Sample is the reciprocal of the serum dilution determined to contain one $TCID_{50}$.

4.8 If CPE is difficult to interpret, an FAT may be conducted as follows:

4.8.1 Decant media from the MDBK Plate.

4.8.2 Fill wells with 80% Acetone.

4.8.3 Incubate at RT for 15 ± 5 minutes.

4.8.4 Decant the 80% Acetone from the MDBK Plate and air dry at RT.

4.8.5 Pipette 35 μl of Swine Anti-Pseudorabies Fluorescein Isothiocyanate Labeled Conjugate into all wells. Incubate for 45 ± 15 minutes at RT.

4.8.6 Rinse by filling the wells completely in D-PBS, allow to stand for 5 ± 2 minutes, and decant.

4.8.7 Repeat for a total of 2 washes.

4.8.8 Dip the plate in distilled water, decant, and allow to air dry or dry at 36^0 ± 2^0C.

4.8.9 Examine wells with a fluorescent microscope.

4.8.10 A well is considered positive if typical nuclear, apple-green fluorescence is observed.

4.8.11 Record and calculate as in **4.6.2** through **4.6.4.**

5. Interpretation of the test results

5.1 The test is invalid if visible contamination or serum toxicity is observed in $\geq$2 wells of all dilutions of a Test Serum Sample.

5.2 The test is invalid if CPE or fluorescence is observed in any of the control wells.

5.3 For a valid assay, the Virus Back Titration must have between 50-300 $TCID_{50}$/100 μl.

6. Report of test results

6.1 Record all test results on the test record.

7. References

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7.3 Finney DJ. Statistical Method in Biological Assay. 3rd ed. 1978. Charles Griffin and Company, London.

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7.5 Oren SL, Swenson SL, Kinker DR, Hill HT, et al. Evaluation of serological pseudorabies tests for the detection of antibodies during early infections. *J Vet Diag Invest* (1993) **5**(4):529-33.

7.6 Parker RA, MA Pallansch. Using the virus challenge dose in the analysis of virus neutralization assays. Statistics in Medicine, (1992) **11**:1253-1262.

7.7 Rose NR, Friedman H, Fahey JL, eds. *Manual of Clinical Laboratory Immunology*. Chapter 11: Neutralization Assays. 1986. ASM, Washington, D.C.

7.8 Weir DM, Ed. *Handbook of Experimental Immunology*. Volume 3. Application of Immunological Methods. Blackwell Scientific Publications, Edinburgh, England. Chapter 40: Immunological Methods in Virology.

8. Changes

8.1 Document has been rewritten to reflect the current format and practices of the Mammalian Virology Section.

Supplemental Assay Method for the Titration of Pseudorabies Virus Neutralizing Antibody

9. Appendix

9.1 Appendix I

Transfer Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A 1:2	TS1	TS1	TS1	TS1	TS1	CC	CC	TS2	TS2	TS2	TS2	TS2
B 1:4												
C 1:8												
D 16												
E 32												
F 64												
G 128												
H 256												

TS= Test Serum
CC= Cell Control